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1. American Journal of Pathology:
   1993 Feb, 142(2):403-412
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Characterization of subcolumnar reserve cells and other epithelia of human uterine cervix

Demonstration of diverse cytokeratin polypeptides in reserve cells

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Summary. We have analyzed the expression of cytokeratin polypeptides in subcolumnar reserve cells of the human uterine endocervical mucosa and the other epithelial cells using immunoperoxidase and immunofluorescence microscopy as well as by applying two-dimensional gel electrophoresis to microdissected cytoskeletal preparations. Endocervical columnar cells were uniformly positive for antibodies directed against the simple epithelium-type cytokeratins nos. 7, 8, 18, and 19, while a variable proportion of these cells was stained by an antibody against cytokeratin no. 4. Reserve cells were not only positive for cytokeratins nos. 8 (weakly and variably) and 19 but were also decorated by antibody KA 1, which reacts with cytokeratins present in stratified squamous epithelia. This last antibody selectively decorated reserve cells even when they were flat and inconspicuous. Antibody KA 1 uniformly stained the ectocervical squamous epithelium, the basal cells of which were also decorated by antibodies directed against cytokeratins nos. 8 (weakly and variably) and 19. Ectocervical suprabasal cells were positive, to a variable extent, for antibodies against cytokeratins nos. 4, 10/11, and 13. Gel electrophoresis revealed the presence of squamous-type cytokeratins nos. 5 and 17 in reserve cell-rich, but not in reserve cell-free, endocervical mucosa. We also analyzed the distribution pattern of these cells, as revealed by antibody KA 1, in the endocervical mucosa of 26 uteri. In all the specimens examined reserve cells were present, but their numbers exhibited considerable variation. In some cases these cells were confined to small islets localized deep within the cervical canal and lacked any continuity with the squamous epithelium. The expression of cytokeratins nos. 5 and 17 in reserve cells indicates that these cells have undergone a low level of squamous differentiation. The additional expression of cytokeratins nos. 8 and 19 in these cells points to a relationship with simple epithelial cells. The present data would seem to favor the view that reserve cells originate in situ from the columnar epithelium; however, this would imply an acquisition of new differentiation properties.

Key words: Intermediate filaments – Cytokeratins – Subcolumnar reserve cell – Metaplasia – Cervical cancer

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Introduction

In the uterine endocervix the surface and glands of the mucosa are lined by a mucus-producing columnar epithelium. Although these mucous cells, which are arranged in a single layer, usually directly border on the basal lamina, a second cell population is sometimes present. This consists of flat or cuboid cells that form a single basal cell layer below the columnar epithelial cells. Long ago these subcolumnar cells were referred to as "reserve cells" by Carmichael and Jeaffreson (1939) who supposed them to be a reserve depot for the regeneration of the mucous-forming epithelium. These investigators also suggested that these cells have a role in the development of squamous metaplasia (Carmichael and Jeaffreson 1941), which may eventually lead to the development of carcinoma-in-situ or invasive squamous cell carcinoma of the cervix (Howard et al. 1949).

The origin of these reserve cells remains an open question: persisting embryonal cells (Meyer

1910), subepithelial stromal cells (Song 1964), monocytes (Reid et al. 1967), immigrating ectocervical squamous cells (Fluhmann 1953), and endocervical mucous cells (Hofbauer 1953) have all been put forward as being the progenitors of reserve cells. Although the epithelial nature of reserve cells would now appear to have established, the exact mechanism of their formation remains uncertain (Lawrence and Shingleton 1980).

Intermediate filament proteins have proved to be efficient tools both for characterizing the differentiation of a particular cell type and, in spite of certain limitations, for establishing the origin of certain cell populations. On the basis of the specific expression of the five intermediate filament classes (cytokeratin, vimentin, desmin, glial filaments, and neurofilaments), the basic routes of differentiation, e.g., epithelial and mesenchymal differentiation, can be distinguished (for review, see Osborn et al. 1983). Furthermore, in the case of epithelial differentiation, the biochemical diversity of epithelium-specific cytokeratins makes it possible to distinguish further subdivisions. Up to now, at least 19 related, but biochemically different, cytokeratain polypeptides have been described, these being expressed in specific patterns in the various types of epithelia. Thus, specific cytokeratin patterns make it possible to discriminate between, for example, squamous and glandular differentiation (Moll et al. 1982; Tseng et al. 1982; Quinlan et al. 1985; Cooper et al. 1985; Moll and Franke 1986).

Several investigators have previously studied various aspects of cytokeratin expression in normal and pathologically altered epithelia of the uterine cervix (Moll et al. 1983; Dixon and Stanley 1984; Puts et al. 1985; Leitner-Gigi et al. 1986; Franke et al. 1986).

In the present study we examined the cytokeratin-polypeptide expression in reserve cells, using a panel of monoclonal cytokeratin antibodies, and by applying two-dimensional gel electrophoresis to microdissected tissue specimens. We were able to demonstrate that subcolumnar reserve cells can be positively identified by their reactivity with antibodies against stratified-squamous-epithelium-type cytokeratins.

Materials and methods

Tissues

Tissues from 26 uteri were obtained at hysterectomy performed as a consequence of nonmalignant disorders (myoma, descensus, etc.). The age of the patients ranged from 30 to 71 years (mean, 47 years). Within about 20 min (maximally 1 h) of removal, during which time the uteri were precooled by placing on crushed ice, 5-mm-thick longitudinal slices comprising the portio and cevical canal were snap-frozen in isopentane precooled with liquid nitrogen. These slices were stored at −80° C in plastic vials to which a few drops of isopentane were added in order to prevent drying. Cryostat sections (4 µm thick) were air-dried, fixed for 10 min in acetone (−20° C), again air-dried and then stored at −20° C. Consecutive fresh longitudinal slices were fixed for 24 h at 4° C in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) and embedded in paraffin (56° C).

Immunocytochemistry

Immunocytochemistry was performed using an indirect immunoperoxidase method and, in some cases, indirect-immunofluorescence microscopy. Frozen sections were rehydrated with phosphate-buffered-saline (PBS) and immediately incubated with the primary antibody for 30 min at room temperature in a humid chamber. The 4 µm-thick paraffin sections cut from the Carnoy-fixed material were deparaffinized in xylol, incubated with Protease Type VII (Sigma, St. Louis, USA; 10 mg/ml in 0.5 M Tris buffer, pH 7.6) for 10 min at 37° C, washed once with cold Tris buffer and twice with PBS and then incubated with the primary antibody as already described. The following primary antibodies against cytokeratins were used:

1. Monoclonal antibody K_G 8.13, which reacts with a broad spectrum of cytokeratin polypeptides (Gigi et al. 1982, cell culture supernatant; used undiluted for immunoperoxidase staining)
2. Monoclonal antibody CK-2, which is specific for the simple epithelium-type cytokeratin no. 18 (Debus et al. 1982; obtained from Boehringer, Mannheim, FRG; diluted 1:20 for immunoperoxidase staining)
3. Monoclonal antibody K_S 8.1, which is directed against the simple epithelium-type cytokeratin no. 8 (R. Hazan and W.W. Franke, unpublished data; Moll et al. 1986; cell culture supernatant; used undiluted for immunofluorescence microscopy, and diluted 1:10 for immunoperoxidase staining)
4. Monoclonal antibody A 53-B/A 2, which is specific for cytokeratin no. 19 (Karsten et al. 1985; obtained from Progen, Heidelberg, FRG; cell culture supernatant; used undiluted for immunofluorescence microscopy and diluted 1:10 for immunoperoxidase staining)
5. Monoclonal antibody CK 7, which is directed against the simple-epithelium-type cytokeratin no. 7 (Tölle et al. 1985; obtained from Amersham-Buchler, Braunschweig, FRG; used diluted 1:3 for immunofluorescence microscopy)
6. Monoclonal antibody KA 1, which reacts with cytokeratin filaments in stratified squamous epithelia (Nagle et al. 1985a, b, 1986; ascites fluid; diluted 1:100 for immunofluorescence microscopy, and 1:25,000 for immunoperoxidase staining)
7. Guinea-pig antibodies against bovine muzzle prekeratins (Franke et al. 1979; guinea-pig serum diluted 1:1,000 for immunoperoxidase staining)
8. Monoclonal antibodies 1C7, 2D7 (van Muijen et al. 1986) and K_S 13.1 (R. Moll, T. Achtstätter, E. Becht, and W.W. Franke, manuscript in preparation; obtained from Progen), which are directed against cytokeratin no. 13 (cell culture supernatants; used undiluted for immunofluorescence microscopy)
9. Monoclonal antibody KA 5, which reacts with cytokeratins nos. 1, 9, 10, and 11 (Nagle et al. 1985a, b, 1986; ascites fluid; diluted 1:100 for immunofluorescence microscopy)
10. Monoclonal antibody 6 B10, which is directed against cytokeratin no. 4 (van Muijen et al. 1986; used undiluted for immunofluorescence microscopy and diluted 1:5 for immunoperoxidase staining).

Negative controls were performed by replacing the primary antibody with PBS or non-relevant monoclonal antibodies; the findings for these controls were consistently negative.

After each antibody-incubation step, the slides were washed three times in PBS. For indirect immunoperoxidase staining, the peroxidase-conjugated secondary antibodies against mouse or guinea-pig immunoglobulins (Dakopatts, Hamburg, FRG) were applied at a dilution of 1:50 in human-AB-serum/PBS (1:3) for 30 min. The substrate solution consisted of 0.6 mg/ml 3,3'-diaminobenzidine (DAB; Merck, Darmstadt, FRG) in PBS supplemented with H_2O_2 (10 µl/ml 3% solution; Merck). The slides were incubated for 10 min, washed with distilled water, then counterstained with Mayer's hematoxylin (Merck; diluted 1:3 in distilled water). In parallel with the immunohistochemical procedures, H & E staining was performed.

For indirect immunofluorescence microscopy, which was applied to acetone-fixed frozen sections, Texas-red-coupled goat antibodies against mouse immunoglobulins (Dianova, Hamburg, FRG) were used as the secondary antibodies. After washing in PBS, the slides were dehydrated by incubation with 95% ethanol, air dried and mounted using Mowiol (Hoechst, Frankfurt-am-Main, FRG).

Microdissection and gel electrophoresis

Regions of endocervical tissue enriched in reserve cells were prepared by microdissecting 20-µm-thick frozen sections under a stereomicroscope as described previously (Moll et al. 1982). Care was taken to exclude metaplastic and ectocervical squamous epithelium, and to minimize the amount of stromal tissue included in the preparation. From the prepared tissue, a cytoskeletal residue was obtained by extraction with a high-salt buffer containing Triton X-100 and subsequent washing with a low-salt buffer (Moll et al. 1982). The remaining pellet was subjected to gel electrophoresis.

Two-dimensional gel electrophoresis was performed using nonequilibrium pH gradient electrophoresis in the first dimension (O'Farrell et al. 1977) and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension (Moll et al. 1982). The gels were stained using the silver staining method described by Oakley et al. (1980). The polypeptide spots were identified by performing co-electrophoresis with marker polypeptides or with samples of known cytokeratin composition (Moll et al. 1982).

Serial sections

Serial sections were cut from Carnoy-fixed material. Every fifth 4-µm-thick section was stained with antibody KA 1 (i.e., corresponding to steps of 20 µm), each series consisted of about 50 steps (i.e., corresponding to a thickness of about 1 mm).

Results

Immunocytochemical and biochemical analysis of cytokeratin polypeptides in subcolumnar reserve cells

Frozen sections of unfixed human uterine cervix were analyzed immunocytochemically in order to investigate their reactivity with various antibodies against cytokeratins. All of the antibodies used reacted exclusively with epithelial cells, resulting in decoration of their cytoplasm; stromal cells remained negative. Apart from the endocervical columnar epithelium and the ectocervical stratified squamous epithelium, we paid special attention to the subcolumnar reserve cells, which were present in a well-developed single-cell layer in some specimens. In addition, we detected some areas exhibiting reserve-cell hyperplasia and, occasionally, squamous metaplasia; the latter type of lesion was not evaluated in the present study (see Leitner-Gigi et al. 1986). The results are summarized in Table 1.

Table 1. Reactivity of cytokeratin antibodies with cell types present in endocervix and ectocervix[a]

Cytokeratin antibody[b]	Endocervix		Ectocervix	
	Columnar cells	Reserve cells	Basal cells	Suprabasal cells
K_G 8.13 (broad spectrum)	+	+	+	+
CK-2 (no. 18)	+	–	–	–
CK 7 (no. 7)	+	–	–	–
K_S 8.1 (no. 8)	+	±[c]	±[d]	–
A 53-B/A 2 (no. 19)	+	+	+	–[e]
KA 1 (stratified squamous epithelia)	–[f]	+	+	+
Guinea-pig antibody[g]	(+)weak	+	+	+
K_S 13.1, 1C7, 2D7 (no. 13)	–	–	–	+
KA 5 (nos. 1, 9, 10, 11)	–	–	–	±het.
6 B10 (no. 4)	±het.	–	–	±het.

het., heterogeneous
[a] As determined by immunoperoxidase staining and/or immunofluorescence microscopy of frozen sections
[b] Cytokeratin specificity is given in parentheses
[c] Variable staining (see text)
[d] Some areas exhibited positive basal cells
[e] Scattered cells were positive
[f] Occasional weak staining (see text)
[g] Raised against bovine muzzle epidermal prekeratins

The broad-spectrum cytokeratin antibody K_G 8.13 stained subcolumnar reserve cells (Fig. 1a) as well as all other epithelial cells, including the columnar cells of the endocervix (Fig. 1a, b) and the entire stratified squamous epithelium of the ectocervix (Fig. 1c).

Antibody CK-2, which is directed against the simple epithelium cytokeratin no. 18, failed to react with subcolumnar reserve cells, but intensely decorated the overlying columnar cells including cytoplasmic processes extending between reserve cells (Fig. 1d), as well as the endocervical colum-

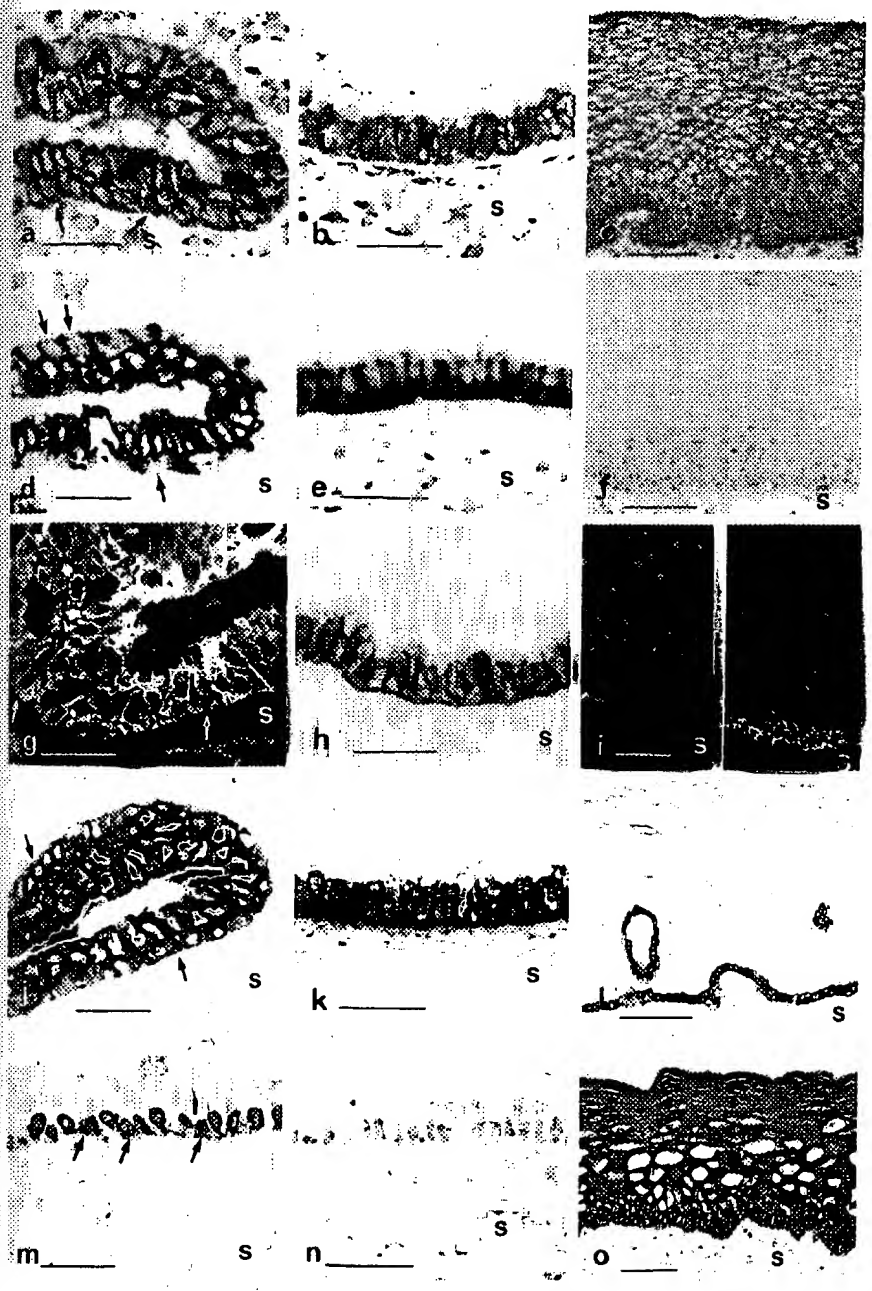

Fig. 1 a–o. Indirect immunoperoxidase staining (**a–f, h, j–o**) and immunofluorescence microscopy (**g, i**) of human endo- and ectocervical mucosa (frozen sections) using various monoclonal antibodies against cytokeratins.
a, d, g, j, m Endocervical columnar epithelium with a single layer of subcolumnar reserve cells (*arrows*);
b, e, h, k, n columnar epithelium devoid of reserve cells;
c, f, i, l, o stratified squamous epithelium of the ectocervix.
a–c Broad spectrum antibody K_C 8.13.
d–f Antibody CK-2 against cytokeratin no. 18; reserve cells are negative (**d**).
g–i Antibody K_S 8.1 against cytokeratin no. 8 (the two pictures in **i** show different specimens). This antibody also showed a weak reaction with some cell nuclei (**i**).
j–l Antibody A 53-B/A 2 against cytokeratin no. 19.
m–o Antibody KA 1, which reacts with cytokeratins of squamous epithelia; note the specific decoration of reserve cells in (**m**).
S, stroma. *Bars* in (**a, b, d, e, g, h, j, k, m, n**), 20 μm; *bars* in (**c, f, i, l, o**) 40 μm

nar epithelium that lacked subcolumnar reserve cells (Fig. 1 e). The ectocervix was completely negative for this antibody (Fig. 1 f).

Antibody CK 7, which is specific for the simple epithelium-type cytokeratin no. 7, also stained columnar cells, but neither reserve cells nor the ectocervical epithelium exhibited any reactivity with this antibody (not shown).

Although antibody K_S 8.1, which is directed against the simple epithelium-type cytokeratin no. 8, stained subcolumnar reserve cells in some specimens, the staining intensity was variable, ranging from weak to moderate (Fig. 1 g). In other specimens, however, these cells were negative for K_S 8.1. In columnar cells, a uniformly intense reaction was observed (Fig. 1 g, h). In some regions of the ectocervical stratified squamous epithelium, antibody K_S 8.1 stained the basal cell layer (Fig. 1 i, right), whereas other areas of this epithelium were negative (Fig. 1 i, left).

Antibody A 53 B/A 2, which reacts with the simple epithelium-type cytokeratin no. 19, uni-

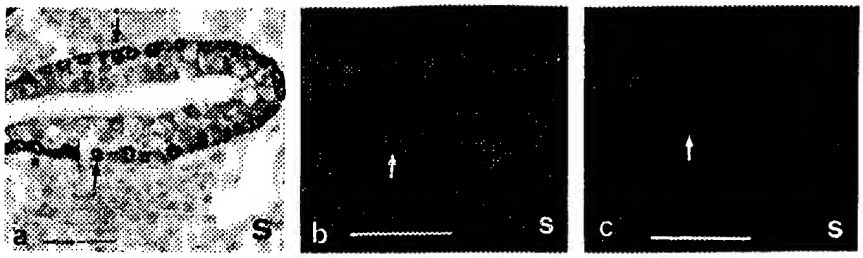

Fig. 2a–c. Immunoperoxidase staining (**a**) and immunofluorescence microscopy (**b, c**) of frozen sections of human endocervix containing columnar epithelium and subcolumnar reserve cells. **a** Polyclonal guinea-pig antibody raised against bovine muzzle prekeratins; note the strong positive reaction of reserve cells. **b** Monoclonal antibody K_S 13.1 against cytokeratin no. 13. **c** Monoclonal antibody KA 5 against cytokeratins nos. 1, 9, 10, 11. *Arrows* denote subcolumnar reserve cells. *S*, stroma. *Bars*, 20 μm

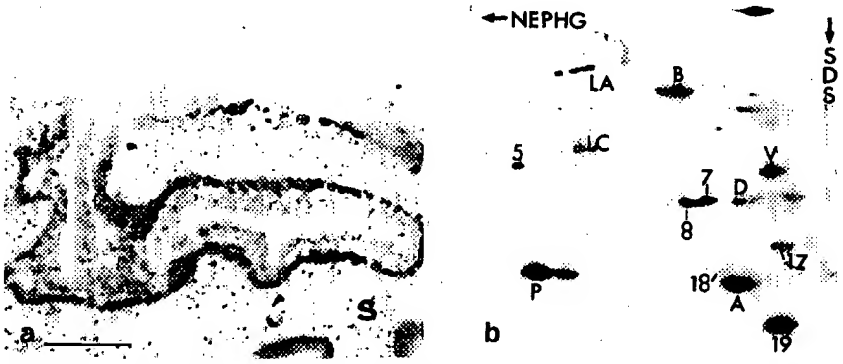

Fig. 3a, b. Biochemical analysis of the cytokeratin polypeptides expressed in endocervical mucosa. **a** Part of the microdissected area analyzed by gel electrophoresis (see **b**) in an indirect immunoperoxidase assay using antibody KA 1. Note the well-developed reserve cells recognized by this antibody. **b** Two-dimensional gel electrophoresis of cytoskeletal proteins. *NEPHG*, direction of the first dimension using nonequilibrium pH gradient electrophoresis (basic polypeptides to the *left*, acidic ones to the *right*); *SDS*, direction of the second dimension in the presence of SDS. Cytokeratins are designated with *arabic numerals* according to the classification outlined in (11). The following polypeptides were added as internal markers for co-electrophoresis: *P*, 3-phosphoglycerokinase (M_r 43000; isoelectric point pH 7.4); *B*, bovine serum albumin (M_r 68000; major variant isoelectric point pH 6.35). *A*, endogeneous actin; *D*, desmin from smooth muscle cells of the fibromuscular stroma; *LA*, lamin A, *LC*, lamin *C* (nuclear envelope proteins). Polypeptides were visualized using highly sensitive silver staining. *S*, stroma. *Bar* in (**a**), 40 μm

formly stained the subcolumnar reserve cells (Fig. 1j), the columnar cells (Fig. 1j, k) and the basal cells of the ectocervical squamous epithelium (Fig. 1l). The suprabasal cells were either unstained or showed staining of single suprabasal cells in some cases. Antibody KA 1, which reacts with cytokeratins of stratified squamous epithelia, specifically and intensely decorated all of the subcolumnar reserve cells in the endocervical epithelium (Fig. 1m). Most columnar cells were completely negative (Fig. 1m, n), although in some areas these cells exhibited a weak staining (not shown). In these areas, reserve cells were usually present below the columnar cells. The stratified squamous epithelium of the ectocervix was uniformly and intensely positive for antibody KA 1 (Fig. 1o). A staining pattern similar to that produced by antibody KA 1 was observed after the application of certain polyclonal guinea-pig antibodies raised against bovine muzzle keratins. The subcolumnar reserve cells were strongly positive (Fig. 2a), as was the ectocervical squamous epithelium (not shown). In contrast, most columnar cells exhibited only weak reactivity (Fig. 2a).

Other antibodies directed against stratified epithelium-type cytokeratins, i.e., 1C7, 2D7 and K_S 13.1 against cytokeratin no. 13 (Fig. 2b) and KA 5 against cytokeratins nos. 1, 9, 10, and 11 (Fig. 2c), showed no reactivity with subcolumnar reserve cells or columnar cells, but they did stain squamous cells of the ectocervix (not shown; Table 1).

In areas with reserve cell hyperplasia, the staining pattern was the same as that observed for one-layered reserve cells.

Since, due to difficulties arising from the immunoblot reactivity of antibody KA 1 (Nagle et al. 1986), the specific cytokeratins recognized by this antibody have yet to be unequivocally established, we also performed a biochemical analysis of the

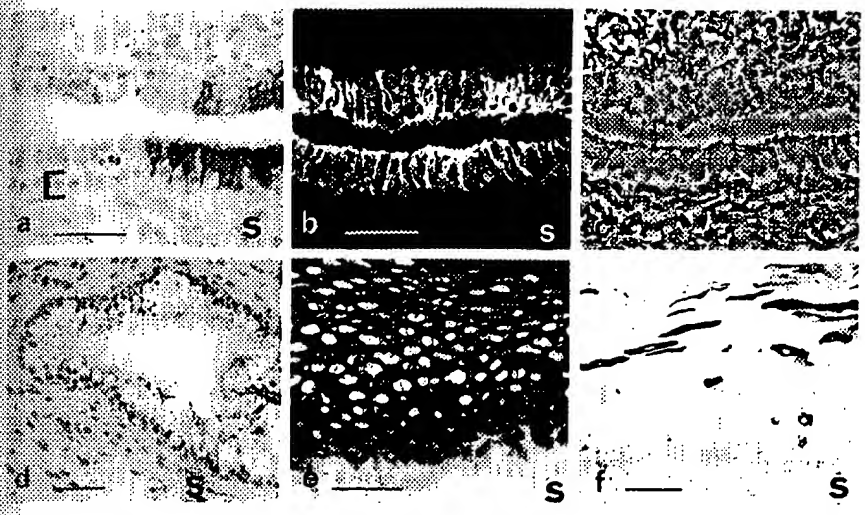

Fig. 4a–f. Indirect immunoperoxidase staining (**a, d–f**) and immunofluorescence microscopy (**b**) of human endo- and ectocervical mucosa (frozen sections) using monoclonal antibody 6 B10 against cytokeratin no. 4. **a** Endocervical mucosa with subcolumnar reserve cells; note the heterogeneous staining of columnar cells. **b–d** Endocervical mucosa containing only columnar epithelium; note positive columnar cells in **b** and negative cells in **d**, (**c**, corresponding phase contrast micrograph to **b**). **e, f** Ectocervical squamous epithelium near the squamoculumnar junction (different specimens); note different extend of staining. *Bracket* denotes reserve cells. *S*, stroma. *Bars* in (**a–d**), 20 μm; *bars* in (**e, f**), 40 μm

cytokeratin-polypeptide pattern of the endocervical mucosa. An area of the endocervical mucosa that is rich in subcolumnar reserve cells – as revealed by staining with antibody KA 1 (Fig. 3a) – was prepared by microdissecting frozen sections consecutive to those stained with KA 1 (Fig. 3a). A cytoskeletal fraction obtained from this tissue preparation (containing columnar cells, subcolumnar reserve cells, and stromal cells) was subjected to two-dimensional gel electrophoresis. Apart from the simple epithelium-type cytokeratins nos. 7, 8, 18, and 19, which are also present in reserve cell-free endocervical mucosa (Moll et al. 1982), significant amounts of the stratified-epithelial cytokeratins nos. 5 and 17 were detected (Fig. 3b).

Antibody 6 B10 is directed against cytokeratin no. 4, which is a typical component of noncornifying stratified squamous epithelia. In the endocervix, the subcolumnar reserve cells were consistently negative for this antibody (Fig. 4a). Among columnar cells, the staining was remarkably heterogeneous, with groups of columnar cells (Fig. 4a) and often entire endocervical glands (Fig. 4b, c) being strongly positive, while columnar cells in other areas were negative (Fig. 4a, d). The proportion of endocervical columnar cells reactive for antibody 6 B10 varied among the specimens examined; for example, in some, the majority of these cells were positive. In the ectocervix, this antibody stained either suprabasal squamous cells in a fairly uniform manner (Fig. 4e), or, in some specimens, it decorated only scattered suprabasal cells (Fig. 4f).

Distribution of subcolumnar reserve cells as revealed by immunocytochemistry

The specific decoration of subcolumnar reserve cells by antibody KA 1 was utilized to study the distribution of these cells within the endocervical mucosa. For this purpose, we used Carnoy-fixed, paraffin-embedded tissue, which yielded very good structural preservation. The reactivity of KA 1 with the reserve cells was not altered by the fixing and embedding procedure; the columnar cells were always negative.

All 26 uteri studied contained at least some subcolumnar reserve cells as demonstrable by KA-1 staining. The immunocytochemical decoration produced by KA 1 allowed these cells to be easily identified even at low magnifications (Fig. 5a). This staining also made it possible to achieve an unequivocal identification of even flat and inconspicuous subcolumnar reserve cells (Fig. 5b, c), which were difficult to detect or to distinguish from stromal cells and tangentially cut columnar cells using H & E staining. All cells exhibiting a clear-cut subcolumnar reserve cell morphology were positive for antibody KA 1. Figure 5 shows a sample containing a small number of subcolumnar reserve cells that are confined to sparsely distributed islets localized deep within the endocervix. In cases with scarce subcolumnar reserve cells the islets detectable in a section were often situated far (up to 0.8 cm) from the squamocolumnar junction. Serial sectioning (Fig. 6) confirmed that these islets,

Fig. 5a–c. Survey micrographs of a longitudinal section through the cervical canal after indirect immunoperoxidase staining using antibody KA 1 (paraffin section). The squamous epithelium and transformation zone are on the *right* (the *arrow* on the *right* indicates the squamocolumnar junction). Subcolumnar reserve cells are only detectable in two distinct, small areas (*framed* in **a**), which are shown at higher magnifications in (**b**) and (**c**). *Bar* in (**a**), 400 µm; *bars* in (**b**, **c**), 80 µm

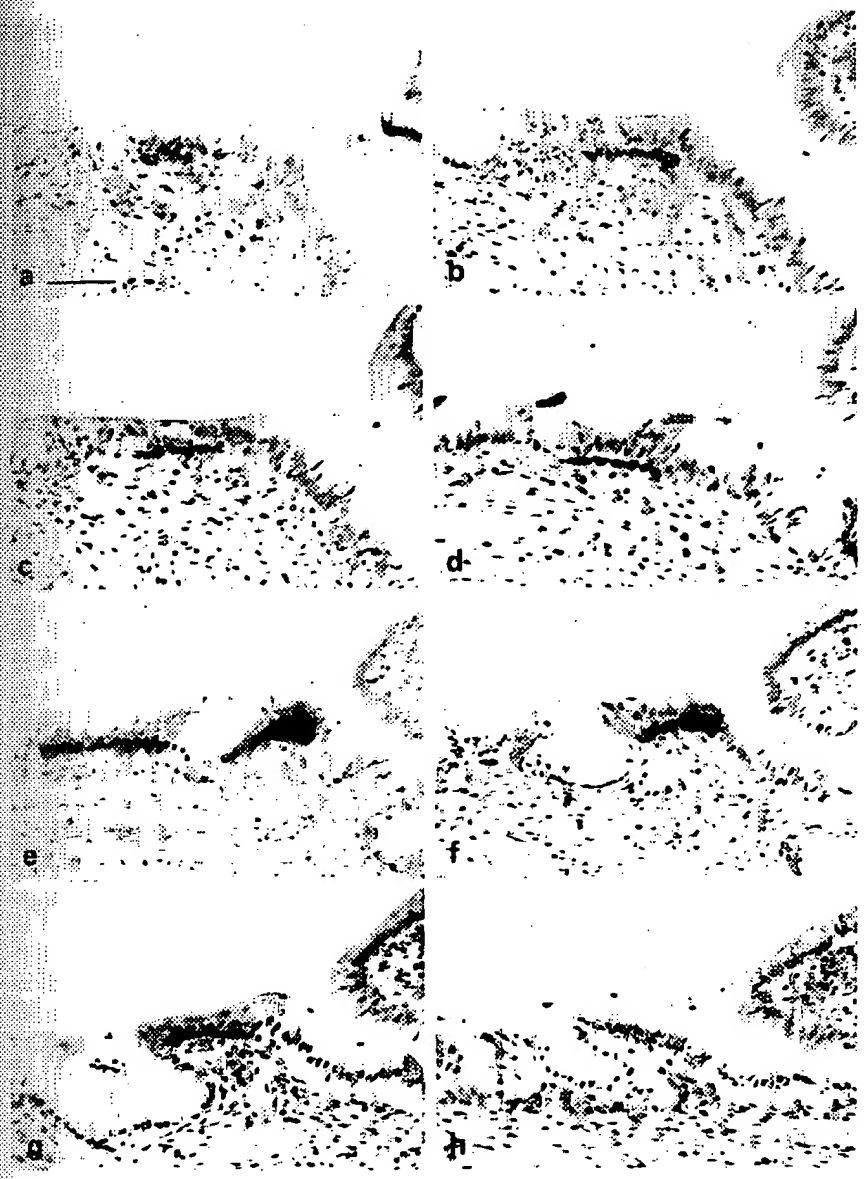

Fig. 6a–h. Serial sections of endocervical mucosa showing the same area in consecutive steps separated by 20 μm; staining with antibody KA 1 (paraffin sections; indirect immunoperoxidase staining). Note that KA 1-positive subcolumnar reserve cells appear in a fairly sharply defined patch beneath the columnar surface epithelium. In the first section of the series (a), only a few reserve cells are present, whereas in the last of the series (h), these cells are completely absent. *Bar*, 40 μm

which were present along the surface epithelium of the endocervix as well as along glands, comprised groups of subcolumnar reserve cells that were completely isolated from other islets and the squamocolumnar junction. In other specimens, we observed extended areas of subcolumnar reserve cells, some of which contained foci of reserve-cell hyperplasia and squamous metaplasia that were also positive for antibody KA 1. Subcolumnar reserve cells mostly appeared as coherent groups.

Discussion

Expression of squamous-typical cytokeratins

In the present study we analyzed the expression of cytokeratin polypeptides in subcolumnar reserve cells of the human endocervical mucosa using a panel of polypeptide-specific, selective antibodies directed against single cytokeratins, and also by applying two-dimensional gel electrophoresis. We were able to show for the first time that reserve cells express certain cytokeratin polypeptides that are not detectable in endocervical columnar cells. One indicator of this difference was the specific immunocytochemical decoration of reserve cells by antibodies such as KA 1. Since unequivocal immunochemical data concerning the cytokeratin polypetides recognized by this antibody have yet to be obtained (Nagle et al. 1986), we used an indirect approach to determine the particular cytokeratins present in subcolumnar reserve cells. Apart from

the endocervix, antibody KA 1 strongly and uniformly stains the cytokeratin filaments present in all layers of nonkeratinizing stratified squamous epithelia and in basal cells of the epidermis, as well as basal cells of the respiratory epithelium and myoepithelial cells (Nagle et al. 1985a, b, 1986).

Biochemical analyses have shown that these types of epithelia have in common the presence of the basic cytokeratin no. 5 along with one or both of the acidic cytokeratins nos. 14 and 17 (Moll et al. 1982, 1983; Quinlan et al. 1985; Cooper et al. 1985; Nagle et al. 1985b). This suggests that antibody KA 1 recognizes one or several of these polypeptides. Further evidence concerning the cytokeratins synthesized in reserve cells was obtained by performing a direct biochemical analysis of the cytoskeletal proteins occurring in endocervical mucosa. In endocervix containing columnar but not reserve cells, only the simple epithelium-type cytokeratins nos. 7, 8, 18, and 19 have previously been detected using gel electrophoresis (Moll et al. 1983). In contrast, our present analysis of cervical mucosa containing a high proportion of subcolumnar reserve cells additionally revealed the presence of cytokeratins nos. 5 and 17. These data strongly suggest that the cytokeratin polypeptides that are expressed in subcolumnar reserve cells are cytokeratins nos. 5 and 17.

Extensive studies of normal epithelia, carcinomas, and cultured epithelial cells have shown that cytokeratin no. 5 along with cytokeratin no. 17 and/or the related cytokeratin no. 14 are consistently expressed in all normal stratified squamous epithelia and squamous cell carcinomas, but are absent in all simple epithelia (Moll et al. 1982, 1986; Tseng et al. 1982; Nelson et al. 1984; Quinlan et al. 1985; Cooper et al. 1985; Nagle et al. 1985b). The only simple epithelium that does express cytokeratins nos. 5, 14, and 17 is that of the amnion, but it is important to note that the amnion epithelium has the potential to form a stratified squamous epithelium (Regauer et al. 1985). On the basis of their tissue distribution, it has been suggested that cytokeratins nos. 5 and 17 may be useful markers of keratinocyte differentiation (Nelson and Sun 1983; Cooper et al. 1985).

It should be borne in mind that a few nonsquamous cell types also express cytokeratins nos. 5 and 17, most notably the myoepithelial cells of various exocrine glands (Moll et al. 1982; Nagle et al. 1985b, 1986); however, it is clear from ultrastructural studies that subcolumnar reserve cells are not related to myoepithelial cells. (Stegner and Beltermann 1969; Gould et al. 1979; Lawrence and Shingleton 1980; Feldman et al. 1984). Rather, subcolumnar reserve cells might be compared to the basal cells of respiratory epithelium, which show a similar cytokeratin phenotype (Blobel et al. 1984; Nagle et al. 1985b). Although not truly squamous, the basal cells of the respiratory epithelium are to some extent related to squamous epithelial cells, because bronchial basal cells may exhibit some ultrastructural features of squamous differentiation, e.g., dense tonofilament bundles (McDowell et al. 1983). Both subcolumnar reserve cells and bronchial basal cells, as well as the basal cells of stratified squamous epithelia have been reported to be selectively positive for the antibody CK-B 1 which is directed against basal/myoepithelial cells (Caselitz et al. 1986); it is possible that this antibody is related to, although not identical with, antibody KA 1. In a previous study it was suggested that subcolumnar reserve cells are of a squamoid nature on the basis of their negative reaction with antibody RGE-53 which is directed against the simple epithelium-type cytokeratin no. 18 (Puts et al. 1985; see also Franke et al. 1986). However, immunocytochemical negativity alone does not represent sufficient evidence for ruling out the presence of a cytokeratin, as this may be due to epitope masking.

Recent ultrastructural investigations (Lawrence and Shingleton 1980; Feldman et al. 1984; Peters 1986) have demonstrated that at least some subcolumnar reserve cells contain cytoplasmic filaments, these having the appearance of perinuclear, bundled tonofilaments as typically encountered in squamous cells. The latter findings have been interpreted as indicating differentiation along a squamous pathway or at least committment towards squamous differentiation (Lawrence and Shingleton 1980). Our findings of cytokeratins typical of squamous cells in reserve cells strongly supports, at the molecular level, the concept of reserve cells having squamous properties.

While cytokeratins nos. 5, 14, and 17 are general markers of squamous epithelia, certain other cytokeratin polypeptides are specific for differentiating squamous cells involved in particular routes of squamous differentiation. These include cytokeratins nos. 10 and 11, which are typical features of keratinizing squamous epithelium, and cytokeratin no. 13, which is a characteristic of differentiating middle and upper layers of nonkeratinizing stratified squamous epithelium. None of these components could be detected in subcolumnar reserve cells. This suggests that reserve cells do not express more advanced forms of squamous differentiation, which is in accordance with morphological findings. In contrast, endocervical squamous metaplasia, which develops from subcolumnar reserve cells (see below), may express such "mature

squamous" cytokeratins, i.e., no. 1 (Loening et al. 1983), nos. 10/11 (Puts et al. 1985; Leitner-Gigi et al. 1986), and no. 13 (Moll et al. 1983; Leitner-Gigi et al. 1986).

In conclusion, the available data concerning their cytokeratin expression and morphology indicate that reserve cells have undergone a squamous differentiation to a limited degree, and therefore, they can be regarded as being immature squamous epithelial cells, or at least facultative progenitor cells of squamous epithelium.

An unexpected expression pattern was observed for cytokeratin no. 4. This polypeptide was found to have a variable and heterogeneous distribution in the columnar epithelium, thus confirming and extending previous data (Franke et al. 1986), but appeared to be absent in reserve cells. Cytokeratin no. 4 is a characteristic and major component of nonkeratinizing stratified squamous epithelia (Moll et al. 1982), but is also expressed in some columnar and unequivocally nonsquamous epithelia, e.g., in some acinar glandular cells of the prostate gland, in pancreatic ductal cells, and in columnar cells of the respiratory epithelium (Van Muijen et al. 1986). Obviously, the expression of cytokeratin no. 4 is not specifically correlated with squamous differentiation, and its significance remains to be established.

Expression of simple-epithelium-type cytokeratins

We were able to show that subcolumnar reserve cells also express some cytokeratins of the simple-epithelium type. Of the four simple-epithelium-type cytokeratins known at present (i.e., nos. 7, 8, 18, 19), cytokeratin no. 19 was clearly demonstrated in reserve cells (in agreement with the data of Leitner-Gigi et al. 1986), while cytokeratin no. 8 seemed to be present only in some of these cells. As we and others have shown, both these cytokeratins are expressed in various simple and glandular epithelia (Moll et al. 1982; Tseng et al. 1982; Quinlan et al. 1985), including endocervical columnar cells (Moll et al. 1983; Dixon and Stanley 1984; Leitner-Gigi et al. 1986). This shows a relationship between endocervical columnar and reserve cells. One should, however, bear in mind that cytokeratin no. 19 is not strictly specific for simple epithelia since it also occurs, usually restricted to the basal layer, in certain stratified squamous epithelia (Moll et al. 1982; Bartek et al. 1986).

The dual characteristics of subcolumnar reserve cells

The data presented so far indicate that subcolumnar reserve cells exhibit squamous characteristics. In addition, they also exhibit certain cytoskeletal features typically found in simple/glandular epithelia including endocervical columnar cells, in agreement with electron-microscopic observations of poorly developed glandular characteristics (Stegner and Beltermann 1969, Gould et al. 1979). Seen together, this would seem to justify the view that they express a dual squamous/simple-epithelial differentiation pattern, albeit at a primitive level.

Interestingly, a dual differentiation pattern is also observable in basal cells of the ectocervical squamous epithelium (Table 1), which have some ultrastructural features similar to those of subcolumnar reserve cells (Ferenczy 1983). However, ectocervical basal cells express some additional squamous markers that are recognized by antibodies K_S 8.12 and K_B 8.37, which are not found in subcolumnar reserve cells (Leitner-Gigi et al. 1986). A comparable dual expression of cytokeratins in a pattern closely resembling that seen in subcolumnar reserve cells has been reported in basal cells of the two-layered epidermis; these cells may give rise both to differentiating keratinocytes and to neuroendocrine (Merkel) cells with simple-epithelial characteristics (Moll et al. 1986).

Histogenesis and fate of subcolumnar reserve cells

What is the significance of these findings with respect to our understanding of the histogenesis and fate of subcolumnar reserve cells? Most previous investigators have found that the incidence of subcolumnar reserve cells is relatively infrequent (Ferenczy 1983). In contrast to purely morphological studies, our immunocytochemical approach facilitated highly reliable identification of reserve cells, as underlined by our being able to detect such cells in all of the 26 uteri studied. This is in accordance with the light-microscopic study of Carmichael and Jeaffreson (1939), who found reserve cells in about 95% of their samples.

Concerning their histogenesis, the possibility that these cells develop from nonepithelial cells such as stromal cells (Song 1964) or monocytes/macrophages (Reid et al. 1967) is almost certainly precluded because they contain cytokeratin filaments and epithelium-specific intermediate filaments, but lack vimentin filaments which are typical features of mesenchymal cells (Caselitz et al. 1986). Our finding that in some uteri, sparsely distributed, very small islets of reserve cells occur deep within the endocervical canal and lack any continuity with the squamocolumnar junction argues against the existence of some form of immigration mechanism as proposed previously (Fluhman 1953), as this would be expected to result in a different distribution pattern, i.e., a much closer spa-

tial relationship between reserve cells and the squamocolumnar junction. In view of their distribution pattern and the fact that certain simple epithelial cytokeratins are features common to both columnar and reserve cells, we favor the concept of an in situ origin of reserve cells from columnar epithelial cells.

The factors that influence the process of reserve cell development are still largely unknown. Gestagen hormones seem to have a promoting influence (Dallenbach-Hellweg 1981), while mechanical lesions of the endocervical mucosa result in the temporary appearance of reserve cells (R. Wagner and H.D. Hiersche, unpublished results). Endocervical reserve cells do not comprise a constant reserve cell population as is evident from their highly variable frequency and their irregular distribution in the endocervix; also, mucous columnar cells have been shown to be capable of self-regeneration (Schellhas 1969). The basal cells of the respiratory epithelium, which exhibit a cytokeratin phenotype similar to that of subcolumnar reserve cells (see above), differ from reserve cells inasmuch as they are a consistent constituent of normal epithelium. This difference may be related to the high level of physical and chemical stress to which the respiratory tract epithelium is subjected. It is possible that the basal (reserve) cells of both the cervical and respiratory epithelium facilitate easy transformation of the vulnerable columnar epithelium to a more resistant squamous epithelium, and may therefore be part of a protection mechanism.

Conclusions

The view that subcolumnar reserve cells originate from columnar cells, when taken together with the present results concerning cytokeratin expression, would imply that, during the formation of reserve cells, the synthesis of stratified-squamous-epithelium-type cytokeratins nos. 5 and 17 is initiated, so that reserve cells acquire a new differentiation property. If metaplasia is defined as being the qualitative transformation of a differentiated epithelium into an epithelium exhibiting a different type of differentiation, the formation of reserve cells must be regarded as being an integral, initial step of the process of squamous metaplasia of the endocervical mucosa, a process that occasionally proceeds via dysplasia to the formation of an in situ and invasive squamous cell carcinoma (Carmichael and Jeaffreson 1941). It appears to be established that squamous metaplasia develops via the proliferation of subcolumnar reserve cells (reserve-cell hyperplasia) that increasingly exhibits the characteristics of mature squamous epithelium (Stegner 1981). The results of the present study suggest that squamous differentiation is already initiated by the appearance of subcolumnar reserve cells, with the expression of certain stratified squamous epithelium-type cytokeratins being maintained during the subsequent steps of squamous metaplasia. Whether the weak KA-1-immunoreactivity occasionally observed in columnar cells, usually in areas containing reserve cells, is related to the process of reserve cell formation remains to be investigated.

When one considers the high incidence of reserve cells in the light of the relatively rare occurrence of squamous metaplasia, it becomes clear that progression to squamous metaplasia is a possible, but apparently uncommon, fate of reserve cells. Indeed, our findings concerning their cytokeratin-polypeptide pattern emphasize the primitive yet dual squamous/simple-epithelial differentiation pattern of these cells. This supports the notion that reserve cells have a bipotent nature (Stegner and Beltermann 1969), so that they have the potential to give rise not only to mature squamous epithelial cells but also (and probably more commonly), to columnar mucous cells. In terms of cytokeratin expression, both pathways would be accompanied by only limited changes. It appears that reserve cells should be considered as being an almost physiological cell type – perhaps as a response to various types of irritation – whose intrinsic capability to undergo squamous differentiation only rarely results in the development of squamous cell carcinoma.

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